

Cell wall inhibitors increase the accumulation of rifampicin in *Mycobacterium tuberculosis*

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Abstract

There is a need for new combination regimens for tuberculosis. Identifying synergistic drug combinations can avoid toxic side effects and reduce treatment times. Using a fluorescent rifampicin conjugate, we demonstrated that synergy between cell wall inhibitors and rifampicin was associated with increased accumulation of rifampicin. Increased accumulation was also associated with increased cellular permeability.

Mycobacterium tuberculosis remains a major public health problem, causing approximately 1.7 million deaths in 2016 [1]. The current treatment regimens for *M. tuberculosis* involve a combination of four drugs, isoniazid (INH), rifampicin (RIF), pyrazinamide and ethambutol. Combination therapy is required to target *M. tuberculosis* in different physiological states. The emergence of resistance against existing drugs emphasizes the need for new therapeutic agents and combination regimens. Synergistic, additive or antagonistic drug interactions occur when the therapeutic activity of drug combinations is greater than, equal to, or less than the sum of the effects of the individual drugs [2]. Identifying synergy between existing tuberculosis (TB) drugs, or those late in development, would allow for new combination regimens that may reduce toxic side effects and the length of treatment.

MmpL3 transports trehalose monomycolates across the cytoplasmic membrane of *M. tuberculosis* and is the target of several families of inhibitors [3–6]. The MmpL3 inhibitor AU1235 has synergy with other TB agents, including RIF, bedaquiline (BDQ) and the β -lactam ampicillin, with a reported fractional inhibitory index (FIC) of 0.5 when used in combination with all three agents [4]. Other MmpL3 inhibitors, including the indole-2-carboxamides and SQ109 (FIC: 0.09), have synergy with RIF *in vitro* and in murine infection models [5, 7]. Synergy with RIF *in vitro* is also observed with other cell wall inhibitors, including ethambutol and inhibitors of Pks13 (FIC: 0.55) [8, 9]. As the target of these inhibitors

is the mycobacterial cell wall, it is likely that the damaged mycolate layer would lead to increased cellular permeability, resulting in increased accumulation and synergy with RIF. We wanted to test the hypothesis that synergy with RIF is at least in part due to increased permeability and intracellular accumulation.

We synthesized a fluorescent derivative of RIF by linking it to fluorescein isothiocyanate (RIF-FITC) (Fig. 1) as described in the supplemental methods (available in the online version of this article). RIF-FITC was active against *M. tuberculosis*, with a minimal inhibitory concentration (MIC) of 0.054 μ M, measured as described in [10, 11], although this is less active than the parent RIF molecule (MIC=0.0060 μ M). RIF-FITC was stable and did not undergo hydrolysis in PBS plus 0.05 % w/v Tween (PBS-Tw) at 37 °C for 3 h, as measured by liquid chromatography mass spectrometry (LCMS). We monitored the accumulation of RIF-FITC in wild-type *M. tuberculosis*. Bacteria were cultured in Middlebrook 7H9 medium plus 10 % OADC supplement (oleic acid, albumin, dextrose and catalase; Becton Dickinson) and 0.05 % w/v Tween 80 to an OD₅₉₀ of 0.6–0.8, washed and resuspended in PBS-Tw to an OD₅₉₀ of 0.6. RIF-FITC was added to cells (0.014–1.4 μ M) and incubated at 37 °C. Samples (3 ml) were harvested, washed in 10 ml PBS-Tw and resuspended in 2 ml PBS-Tw. Bacteria (100 μ l) were dispensed into 96-well black-clear bottom plates and fluorescence was measured at Ex 490 nm/Em 525 nm. We first confirmed that RIF-FITC accumulation could be detected.

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Abbreviations: ATP, adenosine triphosphate; BDQ, bedaquiline; DMSO, dimethyl sulfoxide; EtBr, ethidium bromide; FIC, fractional inhibitory index; FITC, fluorescein isothiocyanate; INH, isoniazid; LCMS, liquid chromatography mass spectrometry; MIC, minimum inhibitory concentration; OD, optical density; RIF, rifampicin; TB, tuberculosis.

Supplementary material is available with the online version of this article.

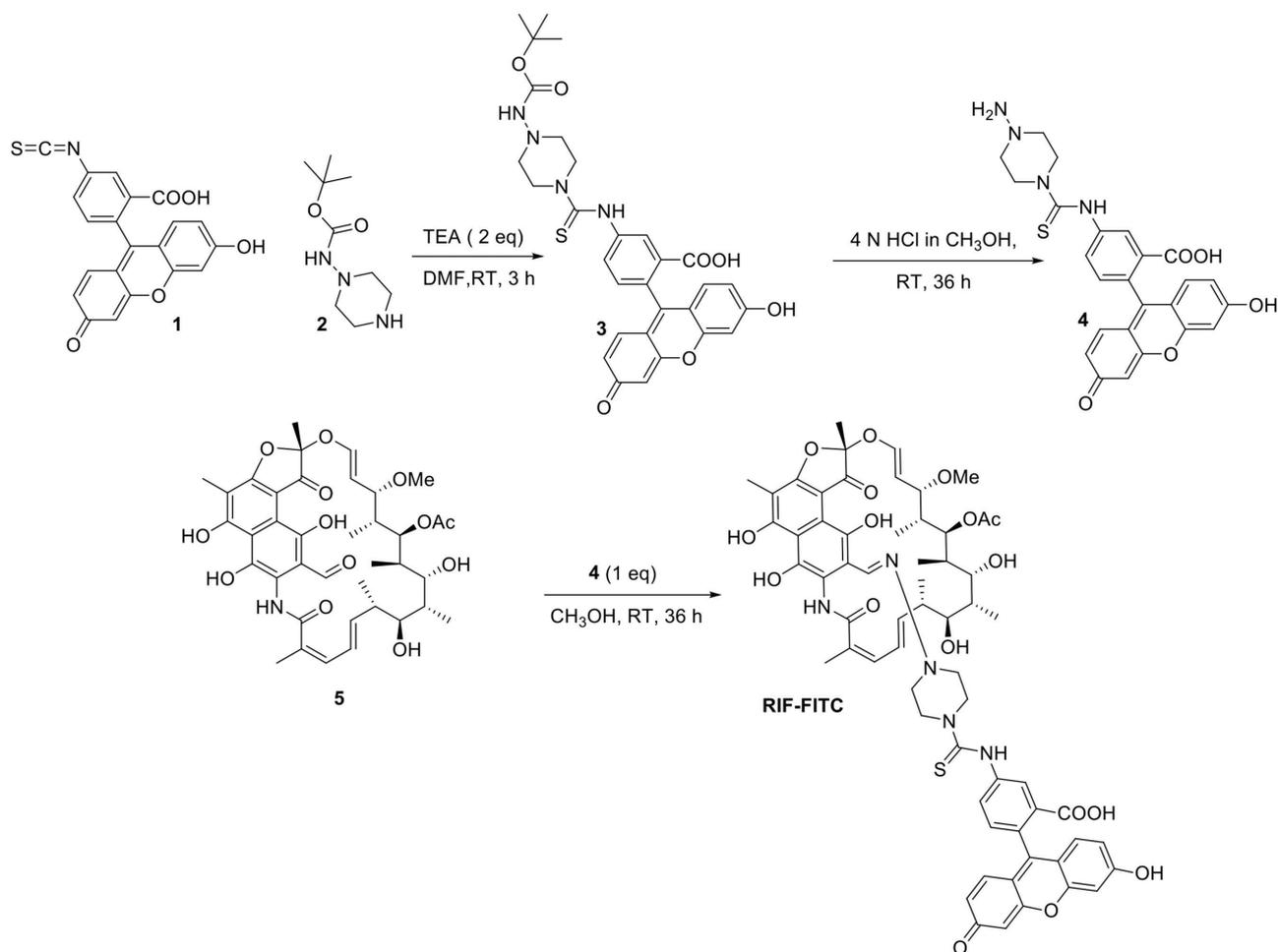


Fig. 1. Schematic for the synthesis of RIF-FITC. TEA, trimethylamine; DMF, dimethylformamide.

A starting concentration of 1.4 μM ($25\times$ MIC) RIF-FITC was required to produce a detectable signal (Fig. 2a). Fluorescence increased in a linear fashion over 3 h (Fig. 2a). Concentrations of $0.25\times$ MIC or $2.5\times$ MIC failed to produce a detectable signal above the background fluorescence (Fig. 2a). All subsequent experiments monitoring RIF-FITC accumulation used a starting concentration of 1.4 μM ($25\times$ MIC).

The MmpL3 inhibitor AU1235 has synergy with RIF *in vitro*, with a reported FIC of 0.5 [4]. We determined whether treatment with AU1235 could affect RIF-FITC accumulation. *M. tuberculosis* was grown to an OD_{590} of 0.3, compound was added and cells were cultured for a further 24 h. RIF-FITC was added to cells and cultures incubated at 37 $^{\circ}\text{C}$. Samples (3 ml) were harvested, washed in 10 ml PBS-Tw and resuspended in 2 ml PBS-Tw. Bacteria (100 μl) were dispensed into 96-well black-clear bottom plates and fluorescence measured at Ex 490 nm/Em 525 nm. The accumulation of RIF-FITC increased twofold after 3 h in the presence of a sub-inhibitory concentration of AU1235 (0.15 μM or $0.25\times$ MIC) (Fig. 2b). At concentrations above the MIC (3 μM or $5\times$ MIC), there was a fourfold increase in intracellular RIF-FITC accumulation (Fig. 2b). Thus, we saw increased accumulation of RIF-FITC

in *M. tuberculosis* following exposure to the MmpL3 inhibitor, AU1235. No aggregation was observed in the cultures after the addition of compounds.

We monitored the accumulation of RIF-FITC in *M. tuberculosis* following exposure to other anti-tubercular agents. We tested RIF-FITC accumulation after 3 h incubation, as we expected this to be at near steady state, as demonstrated in Fig. 2b. At above inhibitory concentrations (i.e. $5\times$ MIC) the cell wall inhibitors, AU1235 (MmpL3 inhibitor) [6], ethambutol (arabinosyl transferase inhibitor) [12], INH (InhA inhibitor) [13] and thiophene-2 (Pks13 inhibitor) [14], resulted in at least a fourfold increase in RIF-FITC accumulation (Fig. 2c). An alternative MmpL3 inhibitor, SQ109, increased RIF-FITC accumulation by twofold (Fig. 2c). The respiratory inhibitors PA-824 [15] and BDQ [16] increased RIF-FITC accumulation by fourfold and twofold, respectively (Fig. 2c). At sub-inhibitory concentrations ($0.25\times$ MIC) AU1235, thiophene-2 and PA-824 all increased RIF-FITC accumulation by twofold (Fig. 2c). INH, ethambutol, SQ109 and BDQ failed to increase RIF-FITC accumulation at $0.25\times$ MIC (Fig. 2c). Other anti-tubercular agents that do not target the cell wall (linezolid, levofloxacin, clarithromycin

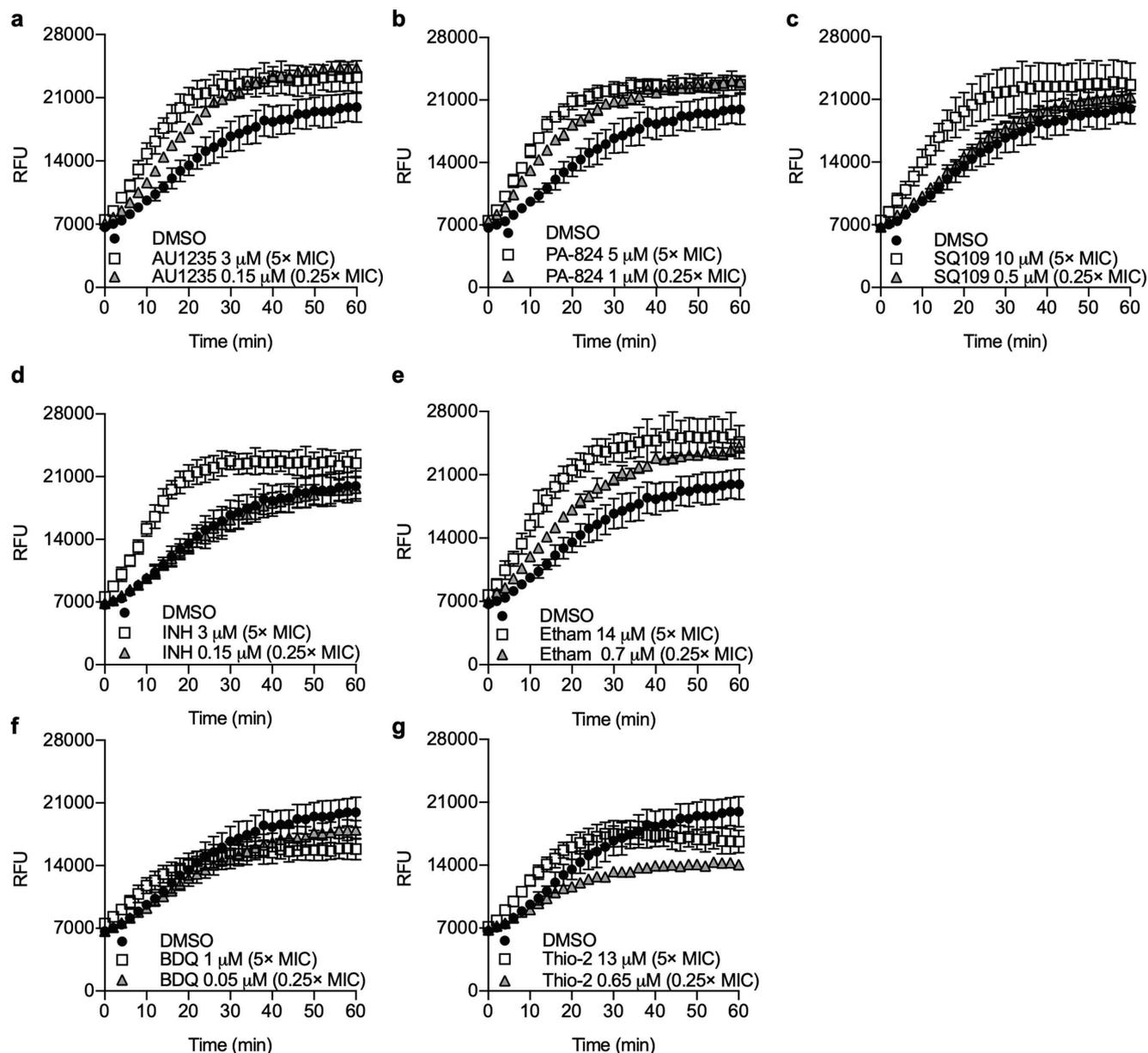


Fig. 2. RIF-FITC accumulates in *M. tuberculosis*. (a) Accumulation of RIF-FITC in *M. tuberculosis*. (b) Accumulation of RIF-FITC in *M. tuberculosis* after pretreatment with 0.15 and 3 μM AU1235 for 24 h ($n=2-3$). (c) Accumulation of RIF-FITC in *M. tuberculosis* after treatment with compounds for 24 h ($n=2-5$). For (b) and (c) RIF-FITC was used at a starting concentration of 1.4 μM (25x MIC). Data are expressed as fold change in RIF-FITC relative to the dimethyl sulfoxide (DMSO)-treated control. The results are the mean \pm standard deviation. The dashed line represents a twofold change relative to DMSO. The MICs for compounds are AU1235 (0.6 μM), SQ109 (2 μM), ethambutol (EMB) (2.8 μM), isoniazid (INH) (0.6 μM), thiophene-2 (Thio-2) (2.6 μM), PA-824 (1 μM), bedaquiline (BDQ) (0.2 μM), linezolid (LZD) (3.2 μM), levofloxacin (LEV) (2.5 μM), clarithromycin (CLA) (1.1 μM) and kanamycin (KAN) (1.3 μM).

and kanamycin) had no effect on RIF-FITC accumulation at 5x MIC (Fig. 2c). In conclusion, a variety of cell wall and respiratory inhibitors increase the accumulation of RIF-FITC in *M. tuberculosis* and a subset of those inhibitors were able to increase RIF-FITC accumulation at sub-inhibitory concentrations.

To determine whether the increased accumulation of RIF-FITC was due to increased permeability, we used the ethidium

bromide (EtBr) assay [17]. *M. tuberculosis* was grown to an OD_{590} of 0.3. Compounds were added and cultures were grown for approximately 24 h; cells were harvested, washed and resuspended in PBS-Tw buffer to an OD_{590} of 0.8. An equal volume of culture was added to 50 μl of PBS-Tw containing 8 $\mu\text{g ml}^{-1}$ EtBr in 96-well plates. Intracellular accumulation of EtBr was monitored at 37 $^{\circ}\text{C}$ using Ex 530 nm/Em 590 nm. AU1235 and PA824, both of which increased

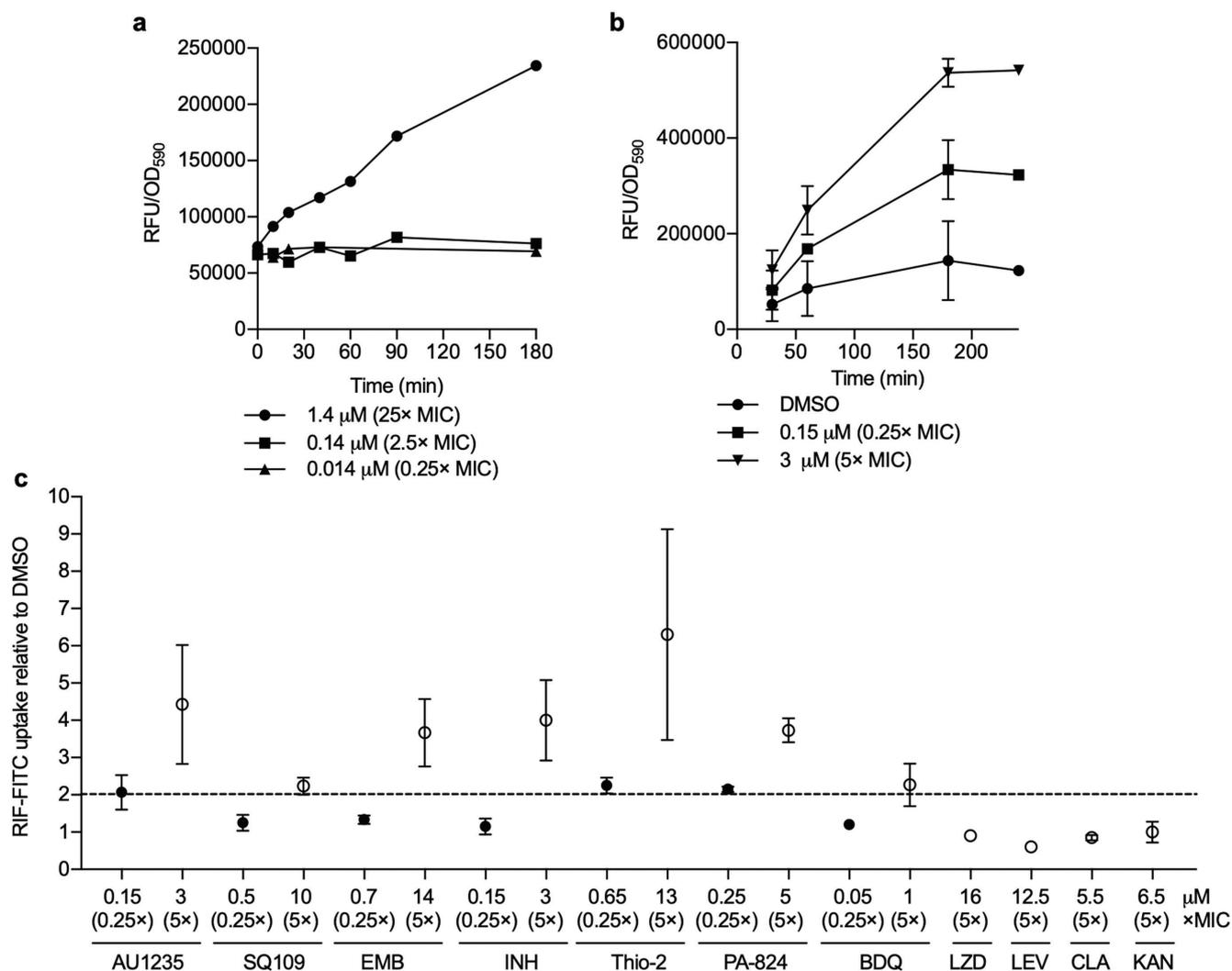


Fig. 3. Cell wall inhibitors increase the permeability of *M. tuberculosis*. *M. tuberculosis* was pretreated with compounds for 24 h. Ethidium bromide uptake was monitored by fluorescence (Ex 530 nm/Em 590 nm). (a) AU1235, (b) PA-824, (c) SQ109, (d) INH, (e) ethambutol, (f) BDQ and (g) thiophene-2. Relative fluorescent units (RFU) from every second minute are presented. The results are the mean \pm standard deviation from technical and biological duplicates ($n=4$).

RIF-FITC accumulation at 5 \times and 0.25 \times MIC, increased EtBr accumulation at 5 \times and 0.25 \times MIC (Fig. 3a, b). At 0.25 \times MIC the rate of accumulation was lower than at 5 \times , although a similar steady state level was reached (Fig. 3a, b). SQ109 and INH, which increased RIF-FITC accumulation at 5 \times MIC but not at 0.25 \times MIC, increased the rate of accumulation and steady state levels of EtBr at 5 \times MIC but not at 0.25 \times MIC (Fig. 3d, e). Ethambutol, which increased RIF-FITC accumulation at 5 \times but not at 0.25 \times MIC, increased EtBr accumulation at both 5 \times and 0.25 \times (Fig. 3f). This correlation suggests that the compounds increase cell wall permeability and/or disrupt efflux, allowing for greater intracellular accumulation of both RIF and EtBr. Interestingly, thiophene-2 and BDQ, which increased RIF-FITC accumulation at 5 \times MIC, resulted in reduced EtBr accumulation as compared to DMSO-treated cells (Fig. 3g, h). This suggests that the mechanisms for increased RIF accumulation for these two compounds are

different and are not related simply to changes in cell wall permeability and/or efflux. Since BDQ disrupts ATP generation, it could have negative effects on ATP-dependent transport mechanisms, which might be independent of changes in cell wall structure.

This study demonstrates that synergistic interactions between RIF and compounds that target proteins involved in cell wall synthesis, including MmpL3 and Pks13, are consistent with disruptions in the cell wall leading to increased cellular permeability and increased accumulation of RIF. Increased accumulation of RIF in ethambutol-treated bacteria is consistent with previous studies that detected intracellular RIF using radio labelling or liquid chromatography [8, 18]. The observation that INH did not increase RIF accumulation at sub-inhibitory concentrations is consistent with previous work showing a lack of synergy between the two agents in

Mycobacterium bovis BCG [18]. However, we demonstrate that at concentrations above the MIC, INH is able to increase the accumulation of RIF. The respiratory inhibitor PA-824 has multiple modes of action, including disruptions in the cell wall [15, 19]. We hypothesize that PA-824-mediated disruptions of the cell wall are responsible for increased permeability and accumulation of RIF. Further studies are required to determine whether synergy between cell wall inhibitors and other compounds is due to increased accumulation and whether increased accumulation translates to improved activity when used in combination.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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